# A STUDY OF THE RELATIONSHIP BETWEEN BONE MORPHOGENETIC PROTEINS AND CRANIOSYNOSTOSIS

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# A STUDY EXAMINING THE RELATIONSHIP BETWEEN BONE MORPHOGENETIC PROTEINS AND CRANIOSYNOSTOSIS

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# TABLE OF CONTENTS


| | Page |
|---|---|
| ACKNOWLEDGEMENTS | iii |
| CONTENTS | |
| I. ABSTRACT | viii |
| II. INTRODUCTION | 1 |
| III. METHODS AND MATERIALS | 7 |
| I. Bone Collection | 7 |
| II. Cell Culture | 7 |
| III. RNA Isolation and PCR | 7 |
| IV. Statistical Analysis | 8 |
| IV. RESULTS | 9 |
| I. Coronal Synostosis | 9 |
| II. Metopic Synostosis | 10 |
| III. Lambdoid Synostosis | 11 |
| IV. Sagittal Synostosis | 13 |
| V. DISCUSSION | 16 |
| VI. CONCLUSION | 20 |
| APPENDIX A: FIGURES | |
| APPENDIX B: SUPPLEMENTARY TABLES | |
| I. Primer Design | 34 |
| II. Patient History | 35 |
| REFERENCES | 36 |

# LIST OF FIGURES


| | Page |
|---|---|
| Figure 1: Schematic of suture placement. | 21 |
| Figure 2: BMP2 mRNA levels in coronal craniosynostosis | 21 |
| Figure 3: BMP4 mRNA levels in coronal craniosynostosis | 22 |
| Figure 4: BMPR1A mRNA levels in patients with coronal craniosynostosis | 22 |
| Figure 5: mRNA levels of BMPR1b in coronal craniosynostosis | 23 |
| Figure 6: GREM1 mRNA levels in patients with coronal craniosynostosis | 23 |
| Figure 7: mRNA levels of NOG in patients with coronal craniosynostosis | 24 |
| Figure 8: BMP2 mRNA levels in patients with metopic synostosis | 24 |
| Figure 9: mRNA levels of BMP4 in patients with metopic synostosis | 25 |
| Figure 10: BMPR1A mRNA levels in metopic synostosis | 25 |
| Figure 11: mRNA levels of BMPR1B in metopic synostosis | 26 |
| Figure 12: mRNA levels of GREM1 in metopic synostosis | 26 |
| Figure 13: Metopic synostosis analysis of NOG mRNA levels | 27 |
| Figure 14: Results from BMP2 mRNA levels in sagittal synostosis | 27 |
| Figure 15: mRNA levels of BMP4 in patients with sagittal synostosis | 28 |
| Figure 16: mRNA levels for BMPR1A in patients with sagittal synostosis | 28 |
| Figure 17: BMPR1B expression in patients with sagittal synostosis | 29 |
| Figure 18: mRNA levels of GREM1 in sagittal synostosis | 29 |
| Figure 19: NOG expression in patients with sagittal synostosis | 30 |
| Figure 20: BMP2 mRNA levels in patients with lambdoid synostosis | 30 |
| Figure 21: BMP4 mRNA levels in lambdoid synostosis | 31 |
| Figure 22: mRNA levels of BMPR1A in lambdoid synostosis | 31 |

Figure 23: BMPR1B mRNA levels in lambdoid synostosis 32

Figure 24: mRNA levels of GREM1 in lambdoid synostosis 32

Figure 25: mRNA levels of NOG in lambdoid synostosis 33

# LIST OF SYMBOLS

| | |
|---|---|
| * | $p<0.05$ compared to normal bone |
| # | $p<0.05$ compared to patent sutures |

# ABSTRACT

**INTRODUCTION:** The high prevalence for craniosynostosis (1) indicates the need for genetic understanding and identification of molecular pathways involved in the premature fusion of the skull sutures. Due to the existing knowledge about bone morphogenetic proteins (BMPs) on ectopic bone formation (2), the role of the BMP family in multiple types of craniosynostosis has long been hypothesized as a key player in the early onset of suture fusion. Based on this hypothesis, the genetic expression of six bone morphogenetic proteins were examined in the four types of synostosis. **METHODS:** Bone collected from patients undergoing corrective craniotomies at Children's Healthcare of Atlanta were received and cells were grown from the bone fragments. From those cells, Real-time PCR was performed to determine the mRNA levels of the predetermined genes. **RESULTS:** Patients expressed individual results based on several factors including suture placement, age at surgery, sex, and predisposition to syndromes known to occur in conjunction with craniosynostosis. The BMPs that were involved in extraneous bone formation and osteoblast hyperactivity were found in high levels in the fused suture bone, while the mRNA levels of the inhibitors of bone formation such as NOG were decreased in fused sutures and exhibited high levels in the patent sutures. **CONCLUSION:** The study further elucidates the role of BMPs in the onset of craniosynostosis and offers insight to the molecular pathways involved.

# INTRODUCTION

At birth, the skull is not uniformly solid, but contains fibrous sutures that allow for necessary brain growth and calvarial expansion. These sutures mineralize over time, allowing for complete skull solidification. Normally, the mineralization occurs after brain growth is complete and development is not affected by the late-fusing sutures. In 1 out of every 2,000 children the suture mineralization occurs prematurely, a malformation known as craniosynostosis (1). The early fusion of sutures often occurs *in utero* or shortly after birth, with complications arising immediately. This premature fusion causes deformities with negative side effects, most notably increased intracranial pressure, vision loss, and mental retardation (3). The effects craniosynostosis can be slowed if action is taken quickly, however the surgical approaches increase the risk that fusion will re-occur. The genetic background of craniosynostosis has yet to be defined and no genetic therapies currently exist.

Craniotomies, procedures in which the skull is removed and reconfigured, are the most common of the surgical techniques used to treat craniosynostosis(3). In this procedure, the surgeon removes the fused suture(s) and rearranges the skull pieces to increase cranial vault volume. This surgery is highly invasive and, due to the natural healing tendencies of children, causes the skull to close the newly formed sutures rapidly in phenomena known as re-synostosis. Because of this re-fusion, the surgery often has to be repeated depending on the age of the patient, need to accommodate additional brain growth, and achievement of an appropriate cosmetic outcome.

The sutures are positioned in four anatomical locations (Figure 1). These sutures are identified as the metopic, lambdoid, sagittal, and coronal sutures. Fusion of the

coronal suture is called anterior plagiocephaly and is located at the crown of the head at the top of the prefrontal cortex. The metopic suture, or trigonocephaly when fused, is at the very top of the skull in between the eyebrows. The lambdoid suture is located in the back of the skull, tracing the occipital lobe, and its' fusion is called posterior plagiocephaly. Lastly, the sagittal suture fusion, or scaphocephaly, occurs along the top of the skull, where the two sides of the brain meet (4). Another phenomenon found with craniosynostosis is pan-synostosis, in which all sutures are fused and the entire skull is solid. In this study, all sutures were examined independent of location. Each type of synostosis has a different prevalence and associated risk factors.

Craniosynostosis is divided into two categories: syndromic and non-syndromic. Syndromic craniosynostosis is presented with other malformations concurrent with an identified gene mutation and has a prevalence of approximately 20% of all craniosynostosis cases (5). The syndromes that are present with craniosynostosis often involve malformations of the limbs and facial features, most notably palate development. Research does not yet exist on the relationship between malformations in non-syndromic craniosynostosis and craniosynostotic patients with deformation of the limbs and palates. The genetic background of craniosynostosis is undefined because of this inconsistency.

The etiology of craniosynostosis is complex and still unclear. Research supports the theory that intrauterine constraint is a leading cause, including a study on twinning that cited children with craniosynostosis showed a twinning rate of 2.62 times higher than the rate in normal children (6). Despite the studies citing intrauterine constraint, an analytical study determined that factors related to intrauterine constraint were not significantly important in the onset of craniosynostosis, while acknowledging that

mechanical stimulation can increase osteoblast differentiation (7). These findings, in conjunction with known genetic mutations present with syndromic craniosynostoses (8-11), have led researchers to focus on examining genetic background.

A single mutation encompassing all types of craniosynostosis has not been determined, but it is widely hypothesized that the malformation is a result of both gene mutations and chromosome mutations. Apert's syndrome, a common cause of syndromic craniosynostosis caused by a mutation of FGFR2, appears in approximately 1 in 65,000 cases (12). In a study by Johnson and Wilkie in 2011 found FGFR3 mutations in 25% of all of their cohort, while TWIST1 occurred 19% of the time (5). Certain syndromes are characterized by their mutations, including FGFR1 causing Pfeiffer syndrome (5). The volume of mutations known to cause syndromic mutations suggest that the correct gene has yet to be discovered or that the malformation is a result of a complex network.

The epidemiology of craniosynostosis is now being examined on a large scale. No knowledge exists on alterations in the prevalence of craniosynostosis based on race or geography. The previously-mentioned incidence of craniosynostosis of 1 in 2000 applies relatively uniformly across all populations. Non-syndromic craniosynostosis occurs more frequently than syndromic (13), but little information is known on the genetic or environmental mechanisms involved in craniosynostosis incidence.

In hopes of understanding the cause of craniosynostosis, several animal models have been developed. Mice models are common models of genetic mutations that present themselves in craniosynostosis. Models exist for knockout genes, knock-in genes, and intrauterine constraint. Mouse models exist for many forms of syndromic craniosynostosis (14). A model for Saethre-Chotzen syndrome, uses a knock-out model

for *Twist* (15) and an Fgfr2 model in mice provides an analogous model for Apert's syndrome (14). The size of mice increases the surgical difficulties, most notably on newborns who won't grow to adulthood without surgical intervention(16). While all of the sutures in the human skull fuse at some point in life, in mice only the posterior frontal suture closes while the other sutures maintain patency over the course of the animal's life (14). This suture-difference provides animal models for therapeutic techniques, but can probably not offer any insight into genetic mechanisms.

Rats also have several syndromic craniosynostosis models. These models show mutations of Noggin and Runx2 expression that are similar to human suture fusion, and are wide-spread models for current therapeutic techniques (1). While rats and mice are usually used to test protein mutations, a rabbit model is the most common to test mechanically induced craniosynostosis, providing insight to intrauterine constraint (16).

Bone morphogenetic proteins (BMPs) were first identified for their ability to induce bone and cartilage formation. BMPs belong to the TGF-β superfamily and are a large number of soluble proteins regulated by numerous inhibitors, receptors, and intracellular molecules (17). Members of the bone morphogenetic protein family have been identified as key players in skull morphogenesis (18), implying that they have an important role in skull development and subsequent suture fusion.

FGFR2, fibroblast growth factor 2, is the identified genetic mutation that causes Apert's. It plays an important role in osteoblast differentiation through regulation of the amount of fibroblast growth factor proteins, a protein important for cell growth. Other known genetic mutations have been identified in TWIST, MSX-2, and FGFR genes (11), all of which are related to BMPs, osteogenic differentiation, and bone formation at the

neural plate border (11). BMP2 is an activator for the MSX-2 gene, of which a mutation has been identified in syndromic craniosynostosis (8). Expression of FGF2 has also been implicated in suture fusion. FGF2 has been found to down-regulate expression of the Bmp antagonist Noggin in coronal dural cells and osteoblasts (8), and is regulated by tyrosine kinase receptors. A gain-of-function mutation in the tyrosine-kinase receptors has been linked to suture fusion in murine models (14). Studies have shown that Noggin, a potent BMP antagonist that prevents osteoblast differentiation (19), supporting the hypothesis that it is important in suture patency. The knowledge that exists about MSX-2, TWIST, and FGFR2 implies that an interaction of the three molecules alters BMP signaling and osteoblast fate in cranial sutures (20).

Other members of the bone morphogenetic protein family have important roles in osteogenesis regulation. BMP4 regulates morphology of craniofacial features through its regulation of cranial neural crest cells (21) and has been linked to change the shape of mice skulls and facial features (21). BMP receptors bind to specific bone morphogenetic protein molecules, resulting in either activation or inactivation of BMP signal transduction (22). BMP signaling is regulated by diverse number of receptors. BMPR1A, bone morphogenetic receptor 1a, has been implicated in facial development, specifically palatal development (23). BMPR1B functions are similar to those of BMPR1A, but the molecule more involved in apoptosis of chondrocytes than BMPR1A, which is more important in differentiation (22). While the aforementioned genes focus mainly on increased osteoblastic activity in the fused suture, BMP antagonists focus on inhibiting BMP activity. In mice, the BMP antagonist Noggin has been shown to be almost entirely

absent is fusing sutures (20), suggesting that BMP antagonists like Noggin and Gremlin1 are important in the longevity suture patency.

Due to the elusive nature of the genetic factors that result in craniosynostosis, a need for genetic mapping is evident. By creating a large-scale analysis of the genetic components that surround the closing suture, possible therapies can be more easily created. The mechanisms involved in the premature suture fusion are complex and, as a network, create a malformation that dramatically decreases patients' quality of life. An analysis of several genes offers potential insight to novel treatments.

# METHODS AND MATERIALS

## I. Bone Collection

Bone samples were received from Children's Healthcare of Atlanta (CHOA) under IRB approval from both CHOA and Georgia Tech. The bone was collected from donors undergoing corrective surgery for craniosynostosis and was immediately placed in media for preservation.

## II. Cell Culture

Cells were grown from three bone samples on the skull: open (patent) suture, the fused suture, and a piece of normal bone. From the three types of bone, osteoblasts were isolated and excess soft tissues were removed. The bone was cut 1-2mm wide and was thrice washed in Hank's balanced salt solution (HBSS) with penicillin-streptomycin. For 15 minutes, the bone was digested at $37^{O}$ C with 0.25% trypsin-ethylenediaminetraacetic acid (EDTA; Invitrogen, CA, USA) and the digestion was discarded. The bone was then cut into 2 mm x 2mm fragments and placed in Dulbecco's modified Eagle medium (DMEM; cellgro, Mediatech, Inc, VA, USA) with 1% penicillin-streptomycin (Invitrogen) and 10% fetal bovine serum (Hyclone, UT, USA). The cells were sub-passaged and first passage cells were used for the experiments.

## III. RNA Isolation and PCR

RNA was extracted from the cells to create cDNA for PCR and subsequent gene analysis. The cells were frozen in liquid nitrogen and placed in TRIzol ® (Life Technologies, Carlsbad, CA), following the manufacturer's directions. RNA purification was done using Qiaquick RNeasy mini-kit (Qiagen, Valencia, CA) and was

quantified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher, Wilmington, DE). RNA was reverse transcribed in quantities from 125ng to 500ng, with the ratio used to create cDNA remaining constant. cDNA was produced with a High Capacity cDNA Kit (Life Technologies) and was diluted for PCR. This diluted cDNA, along with primers designed on the Beacon designer software and purchased from Eurofins MWG Operon (Huntsville, AL), were used to measure expression of the bone morphogenetic proteins through primers in Supplementary Table 1. Pre-designed primers for *Noggin* were used (Qiagen, Valencia, CA). This expression was quantified through real-time PCR in StepOnePlus Real-Time PCR System and *Power* Sybr® Green Master Mix (Applied Biosystems). All mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**IV. Statistical Analysis**

Data are presented as normalized to normal bone mRNA levels. Statistical significance was determined using a Mann-Whitney U test with a significance threshold of $p < 0.05$. ANOVAs were performed with post-hoc tests of Bonferroni's correction to the Student's t-test.

# RESULTS

## I. Coronal Synostosis

Patient histories for coronal synostosis samples included in this study are shown in Supplemental Table 2. The majority of the patients were female (n=7 of 8) and most(n=5 of 8) had family histories of syndromic craniosynostosis or histories of suspected syndromic craniosynostosis. The mean age of the patients was 8.88 ± 2.64 months, with ages ranging from 2 to 24 months at time of surgery. Patent sutures were not collected for three patients. mRNA levels for BMP2 in patent sutures were not significantly different than the normal bone and were lower than fused sutures in all of the patients (Figure 2). Seven of the eight patients had higher levels of BMP2 mRNA in fused sutures compared to normal bone, while all of the patients were upregulated in comparison to patent suture. BMP4 (Figure 3) showed that, in patent sutures, three of the eight patients had higher mRNA levels in patent sutures compared to normal bone, while two of the eight were not significantly different. Fused sutures exhibited higher levels of mRNA of BMP4 in four patients compared to normal bone and in two patients compared to patent suture. BMPR1A mRNA levels were higher in patent sutures in two patients compared to normal bone and two patients compared to fused suture (Figure 4). The fused suture had higher mRNA levels of BMPR1A in two patients compared to both normal bone and patent suture. BMPR1B expression (Figure 5) elucidated patent suture expression was downregulated compared to normal bone in four out of the five donors with collected patent sutures. The fused suture had lower levels of mRNA compared to normal bone in six of the eight patients and was upregulated compared to the patent suture expression in two patients. The BMP antagonist GREM1 had higher mRNA levels

in patent sutures compared to normal bone in two of the eight patients and lower levels in one (Figure 6). The fused suture bone produced lower levels for four patients compared to both normal bone and patent suture. The fused suture bone expression was upregulated in one patient compared to patent suture and two patients compared to normal suture bone. Lastly, the BMP antagonist NOG expressed higher levels of mRNA in patent suture compared to normal bone in all five patients with collected patent suture (Figure 7). NOG was lower in the fused suture of seven patients compared to normal bone and six patients compared to patent suture.

## II. Metopic Synostosis

Patient history for metopic synostosis can be seen in Supplementary Table 3. Of the patients, all were male (n=8 of 8) and 25% had either a familial history of syndromic craniosynostosis or syndromic craniosynostosis (n=2 of 8). The average age of the patients at time of metopic surgery was 11.43 ± 8.09 months, with patient age ranging from 3 to 28 months. Of the mRNA levels of BMP2 in metopic patients (Figure 8), four of the patients expressed lower levels of patent bone expression compared to normal bone, with one patient exhibiting higher BMP2 mRNA levels compared to both normal and fused suture bone. Three patients had lower mRNA levels of patent suture expression in comparison to fused suture bone. The fused suture bone resulted in lower levels in four patients compared to normal bone and higher levels in two patients. BMP4 mRNA levels (Figure 9) were lower levels in patent sutures of two patients compared to normal bone and four patients compared to fused suture. The fused suture bone exhibited higher mRNA levels in four patients and lower mRNA levels in two patients compared to normal bone. BMPR1A mRNA in patent sutures (Figure 10) was lower in one donor and

was upregulated in two donors compared to normal bone. The patent suture had higher mRNA levels in two patients compared to both normal bone and fused suture. The fused suture levels of mRNA were downregulated in three and two patients compared to normal and patent suture, respectively. BMPR1A mRNA was upregulated in two patients compared to both normal and patent suture. BMPR1B mRNA levels (Figure 11) in patent sutures measured higher mRNA levels in four patients compared to normal bone and lower mRNA levels in one patient compared to normal bone. The fused suture demonstrated lower levels of BMP1B mRNA in three patients compared to normal bone and six patients compared to patent suture. The fused suture expressed more mRNA in two patients compared to both normal bone and patent suture. The BMP antagonist (Figure 12) expressed higher mRNA levels in patent suture of five patients compared to both normal bone and fused suture bone. The patent suture expression was downregulated in two patients compared to normal bone and three patients compared to fused suture bone. Additionally, NOG (Figure 13) had higher mRNA levels in the patent sutures compared to both normal bone and fused suture bone in seven of the patients. The fused sutures expressed less mRNA in six of the donors compared to normal bone and seven donors compared to patent suture.

### I. Sagittal Synostosis

Sagittal suture patient histories are located in Supplementary Table 4. The majority of the donors were male (n=5 of 7). Of the six patients, none had familial histories of syndromic craniosynostosis or identified syndromic craniosynostosis. The average age of patients at time of surgery was 6.17 ± 2.59 months, with patient age ranging from 1 to 19 months. One of the patients did not donate patent suture. BMP2

mRNA levels (Figure 14) demonstrated lower levels in two patients compared to normal bone and lower mRNA levels in four patients compared to fused suture bone. BMP2 was upregulated in patent suture compared to normal bone and fused suture in one patient. The fused suture levels were upregulated compared to normal bone and patent suture in four patients. The fused suture exhibited downregulated BMP2 mRNA in one patient compared to patent suture. Figure 15 shows BMP4 mRNA levels. The patent suture revealed less mRNA in two sutures compared to fused suture bone, and higher amounts of BMP2 mRNA in two patients compared to both normal bone and fused suture bone. The fused suture had higher mRNA levels in three patients compared to normal bone, with less BMP4 mRNA in one patient. Bone morphogenetic protein receptor 1A, BMPR1A, mRNA levels in patent sutures were higher in one patient compared to normal bone, with all other differences compared to normal bone being insignificant (Figure 16). Patent suture mRNA compared to fused suture mRNA was downregulated in two patients, with other differences lacking significance. Fused suture mRNA was upregulated in three patients compared to normal bone, two patients compared to patent suture, and no significant mRNA differences when compared to either normal bone or patent suture. BMPR1B (Figure 17) demonstrated significantly less mRNA in patent suture in one patient compared to normal bone and significantly higher mRNA levels in one donor. Compared to fused suture bone, one patient supplied significantly lower levels of BMPR1B mRNA in patent suture, while two patients supplied significantly lower levels compared to fused suture. BMPR1B mRNA was upregulated in one patient compared to both normal bone and fused suture. Fused sutures expressed higher levels of BMPR1B mRNA in two donors compared to normal bone and patent suture, with less

mRNA expressed in one donor's fused suture compared to patent suture. The BMP antagonist, GREM1, showed significant higher levels of patent suture expression compared to normal bone, with no donors expressing significant lower levels (Figure 18). The patent suture GREM1 mRNA levels were downregulated in two patients compared to fused bone and upregulated in three donors compared to fused suture. Fused sutures expressed less GREM1 mRNA in three patients and higher levels in three patients compared to normal bone. The fused suture demonstrated lower levels of mRNA compared to patent suture in three patients and higher levels in two. NOG mRNA levels (Figure 19) were analyzed, with patent sutures expressing significantly less NOG in five patients and higher mRNA levels in one patient compared to normal bone. The patent suture exhibited increased GREM1 mRNA in five patients compared to fused suture and lower levels in one. Fused sutures showed significantly higher mRNA levels in one donor compared to normal bone.

## II. Lambdoid Synostosis

Patient histories for the analyzed population of lambdoid synostosis (n=4) can be seen in Supplementary Table 5. All of the donors tested were female (n=4), with a minority (n=1) representing syndromic cases. The average age of the patients at time of corrective surgery was 12.75 ± 5.62 months, with the ages ranging from 7 to 20 months. BMP2 mRNA levels (Figure 20) were higher in patent sutures in three patients compared to normal bone, with significantly downregulated mRNA levels in one patient. Patent sutures expressed lower levels of BMP2 mRNA in one patient and higher levels in one patient compared to fused suture bone. The fused suture expressed higher levels of BMP2 mRNA in three patients compared to normal bone and one patient compared to patent

sutures. The fused sutures indicated lower levels of BMP2 in one patient compared patent suture. BMP4 mRNA (Figure 21) in the patent suture was significantly lower in one patient and higher in one patient compared to normal bone cells. The patent sutures had lower BMP4 in three patients compared to normal bone and higher in one patient. Fused sutures had higher levels of BMP4 mRNA in three of the donors compared to normal bone and patent sutures, with the other donor expressing lower levels of mRNA compared to both normal and patent suture cells. The mRNA levels of BMPR1A (Figure 22) demonstrate that patent sutures expressed significant higher levels in two patients compared to normal bone and lower levels of BMPR1A mRNA in three patients compared to fused sutures. Fused sutures had significantly lower levels of BMPR1A compared to normal bone and patent sutures in three patients. BMPR1B mRNA expression in patent suture indicated no significant differences compared to normal bone (Figure 23). The patent sutures expressed lower levels of BMPR1B mRNA in three patients compared to fused sutures. The fused suture mRNA was significantly upregulated in two patients compared to normal bone and significantly lower levels in one patient compared to normal bone and patent sutures. GREM1 mRNA levels (Figure 24) was significantly downregulated in one donor's patent suture compared to the normal bone, while one donor expressed higher mRNA levels compared to normal bone. The patent sutures indicated downregulation in two patients compared to fused sutures. The fused sutures expressed GREM1 in significant upregulation in two patients compared to normal bone and patent sutures. The fused suture expressed lower levels of GREM1 mRNA in one donor compared to the patent suture. The other analyzed BMP antagonist, NOG (Figure 15), elucidates patent suture expression of higher levels of NOG in one

patient compared to normal bone and lower levels in one patient compared to fused suture bone. The patent suture expressed higher levels of NOG in three patients compared to fused sutures. The fused sutures expressed significantly lower levels of NOG mRNA compared to both normal bone and patent sutures.

# DISCUSSION

The purpose of this study was to analyze bone morphogenetic proteins in patients undergoing corrective surgery for craniosynostosis. Current research is highly specific on particular genes or molecules and very rarely on a large scale (24). This study provides data for a large population and helps to better elucidate the phenotype change as well as the possible molecular mechanisms affecting cells in craniosynostosis. Despite a high prevalence of the disease, the majority of literature regarding craniosynostosis is presented from a surgical standpoint. Both the scale of the study and the tested expression of the genes yielded novel results. From the patient history and the gene analysis, an excellent preliminary backstory can be created to explain certain aspects of fused suture formation in craniosynostosis in relation to bone morphogenetic proteins.

The data collected on the patients undergoing surgery for coronal craniosynostosis developed ideas and interactions associated with molecular mechanisms. Interestingly, all samples but one collected were female patients, suggesting a correlation between suture location and gender. The high rate of syndromic synostosis in conjunction with coronal suture fusion is well-researched (25). The syndromes represented in our cohort included Muenke's syndrome, Saethre-Chotzen, Apert's, and Crouzon syndrome. One of the patients suffered from pan-synostosis, a condition in which all the sutures are prematurely fused. The high frequency of syndromic craniosynostosis in coronal suture fusion is supported by existing literature. The majority of patients with coronal synostosis were operated on before twelve months of age, suggesting early onset and expression of the malformation. This early average age of surgery is expected, as coronal suture mutations are usually disruptive in facial development and calvarial expansion. As a

whole, patients with coronal synostosis had a higher incidence of female sex and syndromic craniosynostosis, but a low mean age at operation.

Patients with fused metopic sutures, in comparison to those with fused coronal sutures, underwent corrective surgery at a later age on average, suggesting either later onset of suture fusion or less-obvious and disruptive deformities. In addition to the later surgery time, metopic patients were all male, supporting the hypothesized correlation between male sex and metopic synostosis development (16). Additionally, two patients had identified mutations, with one patient with a microduplication of the chromosome Xq27.3-6 and another with Fragile X syndrome. Chromosome Xq27 has been linked to bone densities of outer limbs (26). This chromosome is a site that has been implicated in the causation of Fragile X syndrome (27), and has been linked to bone, face, and palate development (28). This correlation between chromosome location and metopic suture fusion suggest that chromosome Xq27 should be further explored to either rule it out as a causing factor or develop understanding of its' role in cranial suture fusion. The results of the study of metopic patients suggest a correlation between later age, male sex, and chromosome Xq27 abnormalities and metopic suture fusion.

From our patient samples, four presented lambdoid synostosis. This small number is a relatively good indicator of the actual prevalence of lambdoid synostosis (28). It is unclear if the low enrollment of patients with lambdoid synostosis is a result of the discrepancies between low-prevalence and/or uncommon surgical identification/correction due to a higher age of onset. Lambdoid synostosis had the highest average age of operation, suggesting either later onset or less-obvious suture malformation. Posterior plagiocephaly (lambdoid synostosis) is often confused with

deformational plagiocephaly, usually caused by frequent pressure on the lambdoid suture due to sleeping patterns (29). With this reasoning, later age at time of operation could be attributed to attempts to remedy deformational plagiocephaly before surgical correction of posterior plagiocephaly.

Sagittal suture synostosis is the most common form of synostosis (4). It is important to note that the smaller amount of donors in our study of sagittal synostosis is due to the aggressiveness of surgical therapy. In some surgical cases, the surgeon removed the two strips of bone adjacent to the fused suture, leaving the fused suture intact. In these cases, no fused suture sample was taken, failing to represent the frequency of sagittal synostosis in our population. The data collected showed that the patients with sagittal synostosis had a large age range, but had the lowest mean age at surgery. This low age onset suggests either the critical nature of the premature sagittal suture fusion or early tendencies for fusion. The majority of the patients were male supporting existing research that shows a higher male to female prevalence in sagittal synostosis (scaphocephaly) (4). None of the data collected for sagittal synostosis showed pre-existing syndrome diagnoses at the time of surgery. Our data suggests that sagittal synostosis has earlier ages at operation time and prevalence linked to the male sex.

Our results indicate that BMP2 expression varied largely based on the type of synostosis. High levels of BMP2 are logically present in fused suture based on existing research that suggests that BMP2 is present in ectopic bone formations (2). The metopic sutures yielded different results than the other sutures, with BMP2 being significantly down regulated in both open and fused sutures compared to normal bone. The majority of the patients express higher BMP2 levels in the fused suture than the open suture. High

levels of BMP2 are expected in new bone formation because it is widely known that BMP2 play a key role in osteoblast differentiation and bone formation, including the upper and mid-face (18). Because BMP2 has been found to regulate Msx-2 (2), an identified mutation linked to craniosynostosis (11), the role of the molecule should be examined thoroughly, with its relationship to craniosynostosis being analyzed. Generally, the fused sutures exhibited higher levels of BMP2 mRNA in fused sutures compared to open sutures, with a noticeable amount of higher levels in fused sutures compared to normal bone.

BMP4 yielded different results, despite its similar functions to other members of the BMP family like BMP2 (30). The combination of disruptions in the *in-utero* environment and predisposition to genetic anomalies can significantly discompose facial development (18), suggesting a complex interaction between all the bone morphogenetic protein molecules in developing cranial features. In our study, BMP4 was upregulated in the fused suture compared to patent suture bone, with many patients lacking significant differences between the levels of normal bone mRNA and patent suture mRNA. The lack of similarities between the mRNA levels of BMP2 and BMP4 molecules support findings that BMP4 is present in the mesenchyme, even when BMP2 was absent (18). Our results support the findings BMP4 is involved in morphogenesis (31), with the fused suture and patent sutures having significantly different mRNA levels in the majority of the patients.

BMPR1A, a type I BMP receptor, has been implicated in various osteogenic processes and has subsequently been linked to facial development as a crucial part of mid-face development (32) and is the receptor for BMP2 (33). Because of this

relationship and proximity of facial development to suture fusion, the expression of the mRNA levels of BMPR1A was hypothesized to be important in the network of molecules involved in suture fusion. Lambdoid, metopic, and coronal patients all exhibited lower levels of BMPR1A in fused sutures compared to normal bone, expectedly mimicking the results for BMP2 levels. The results for BMPR1A in patients with sagittal synostosis yield different results, with all of the patients having significant higher levels of activity in fused sutures compared to normal bone activity. Several things may attribute to the anomaly of these results, including, but not limited to, the fact that no patients with sagittal synostosis had either syndromic synostosis or a familial history positive for a syndrome involving craniosynostosis. Additionally, sagittal suture patients had the lowest mean age at time of operation, potentially suggesting a link between receptor presence and age or syndromic mutations.

The relationship between BMPR1B and bone mass is not as clearly defined as the relationship between BMPR1A and BMP2 reception and bone mass (33). BMPR1B levels showed very inconsistent differences through the donors and types of synostosis. Downregulation of BMPR1B in the fused suture compared to the patent suture levels was common, suggesting a lack of receptors in fused sutures. This seems illogical, however, BMPR1B has been shown to have natural differences in expression based on age (34). Our results do not offer clear conclusions regarding the involvement of BMPR1B and cranial suture fusion, suggesting a further need for analysis and explanation.

NOG, a BMP antagonist, has been shown to be present in the patent sutures of patients with craniosynostosis, and, more notably, absent in fusing sutures (20). Our data was consistent with these findings, with very few patients lacking significant

downregulation in fused sutures compared to normal bone. Patients with both metopic and sagittal synostosis also showed a significant higher levels of NOG in patent sutures compared to normal bone, suggesting that Noggin lower levels is directly related to the fusing process. Because BMP inhibitors like NOG block the BMP pathway, their presence suggests of a key role in the bone morphogenetic protein pathway's relationship to suture fate.

GREM1, another BMP antagonist, yields similar data to NOG expression. While the majority of patients had NOG higher levels in patent sutures compared to normal bone, the results of GREM1 are not as consistent. This inconsistency can be attributed to a less direct correlation between GREM1 and key BMP molecules involved in craniofacial development. Both coronal and metopic synostosis showed significant higher levels of GREM1 expression in patent sutures compared to fused sutures. In sagittal and lambdoid patients, however, several patients exhibited the reverse effect, exhibiting higher levels of a BMP antagonist in the fused sutures compared to the patent sutures. This data suggests that the presence BMP antagonists play a large role in keeping sutures open for a longer amount of time.

This study offers several areas for potential expansion. Though the donor population size was large in comparison to most other genetic analyses on craniosynostosis, patient number placed a severe limitation on analyzing data trends. Additionally, data collection placed a strain on the quality of the results. Cells were grown from the bone fragments received, without analyses on the collected bone fragment. Immunohistochemical analysis of the bone samples would offer supporting evidence to the results of the genetic analysis study. Our results characterized the

phenotype of the cells when the suture was already fused, and do not show the cascade of genes that are expressed throughout suture fusion; it would be very difficult to ascertain temporal regulation of genes involved in suture fusion in bone cells. Studies including larger numbers of patients would further elucidate the genetic network that causes premature suture fusion.

# CONCLUSION

Taken together, the data suggests BMP molecules, receptors, and inhibitors are highly regulated in fused sutures, independently of syndromic or non-syndromic craniosynostosis. The results suggest that bone morphogenetic proteins play an important role in the regulation of suture fusion in fused suture sites, as well at the maintenance of patency in normal sutures. Antagonists like Noggin appear to have important activity in both patent sutures and fused sutures. Due to the inconsistencies and maintained individualities in the patients, therapeutic applications of this genetic background are not practical at this level of understanding. Larger-scale studies are necessary to determine the true genetic signatures in craniosynostosis. A different study suggests that the patterns expressed between genes may be the key to therapeutic application, as no genes are currently identified as causing a certain craniosynostosis in a certain suture (35). By widening the lens through which craniosynostosis is examined, it has been suggested that Wnt signaling may pay a more crucial role in preventing premature fusion than BMPs (36).

# APPENDIX A: FIGURES

(For figure keys, please see "List of Symbols")

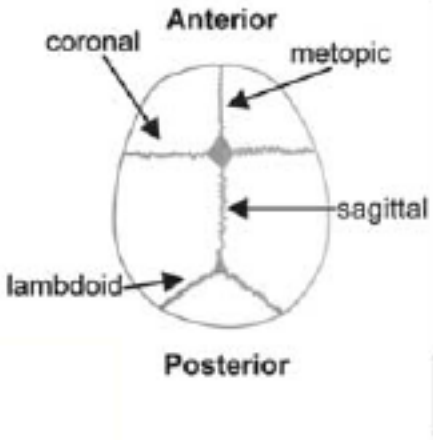


Figure 1. A schematic of suture placement (5).

## I. Analysis of Bone Morphogenetic Proteins In Coronal Craniosynostosis

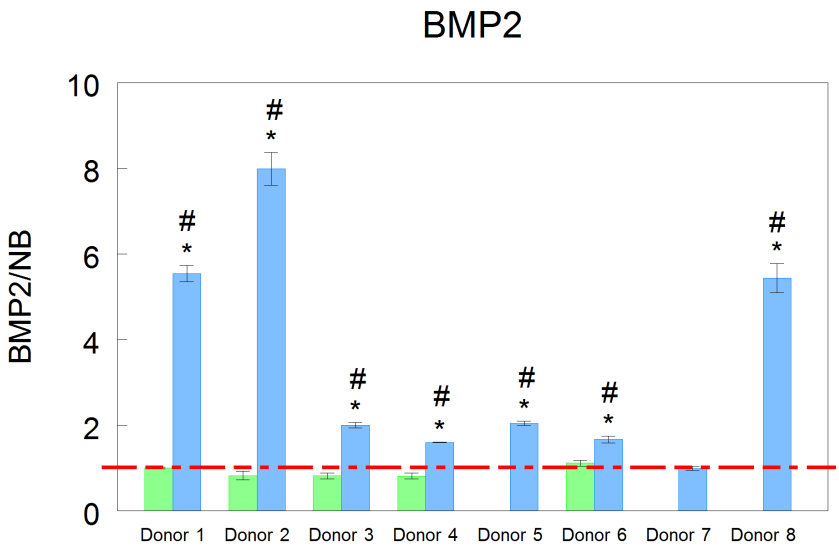


Figure 2. BMP2 mRNA levels in coronal craniosynostosis.

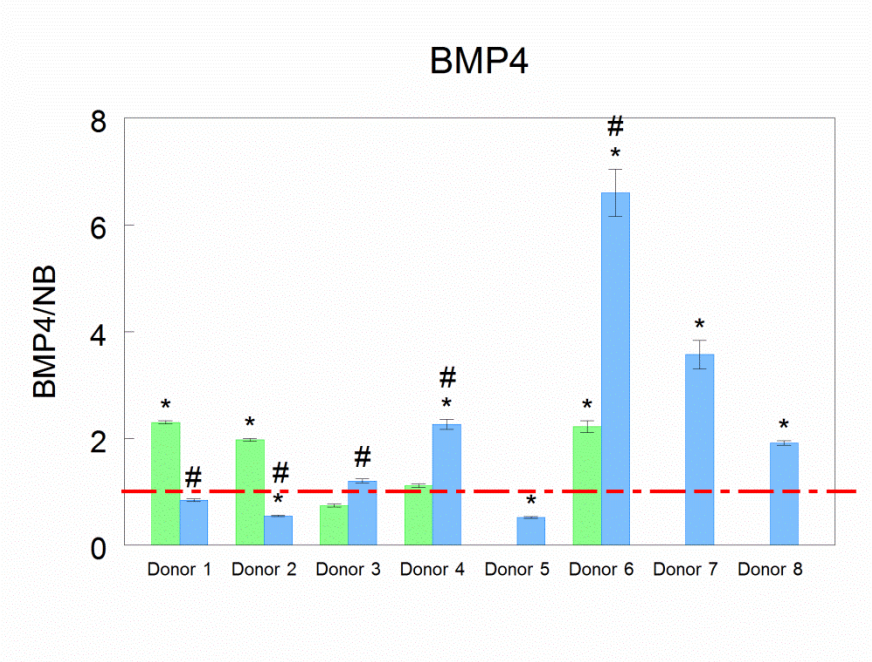


Figure 3. BMP4 mRNA levels in coronal craniosynostosis.

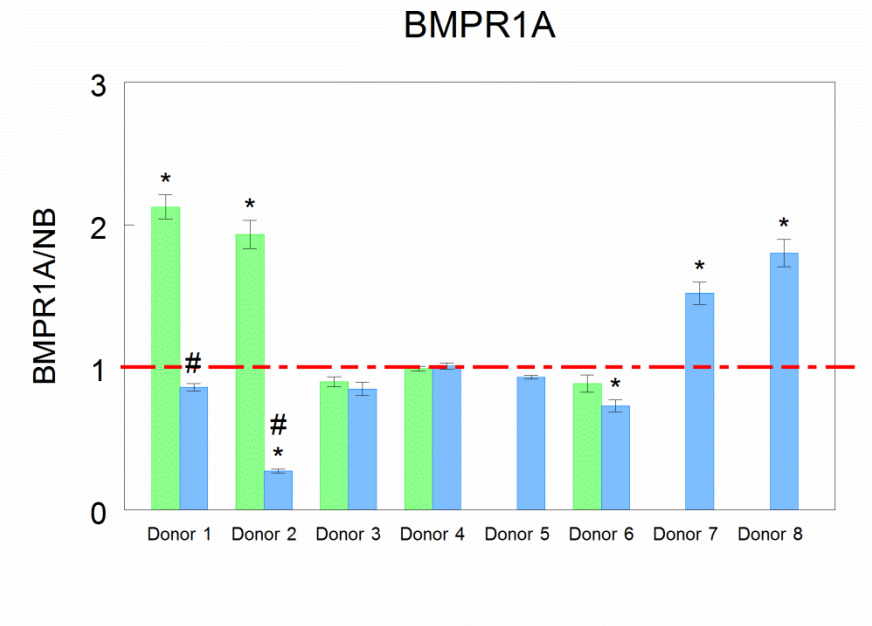


Figure 4. BMPR1A mRNA levels in patients with coronal craniosynostosis.

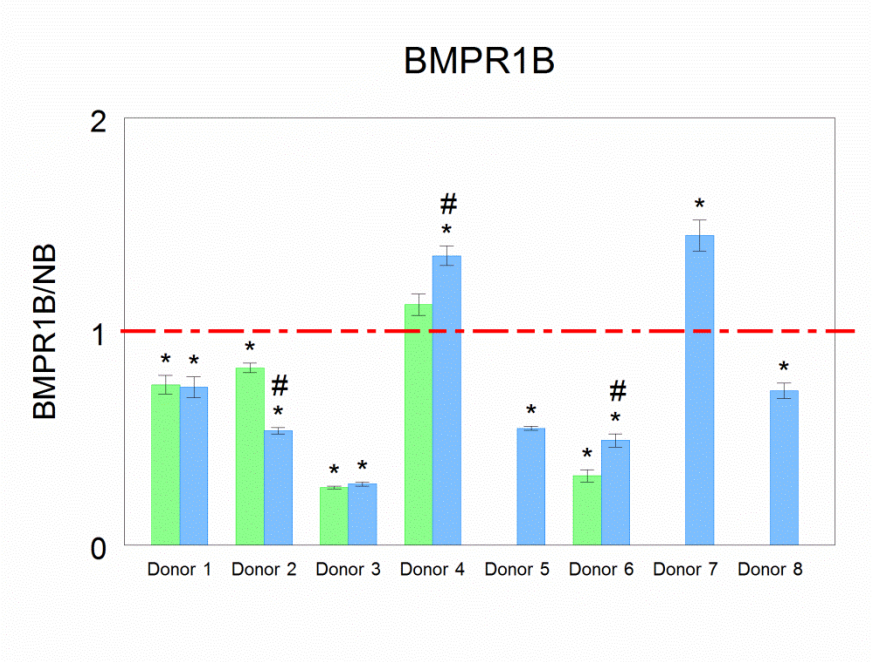


Figure 5. The mRNA levels of BMPR1B in coronal craniosynostosis.

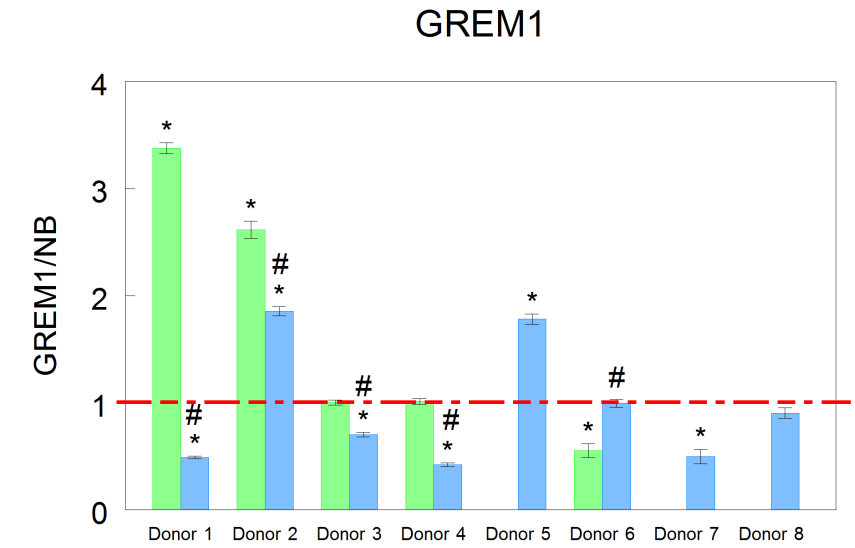


Figure 6. GREM1 mRNA levels in patients with coronal craniosynostosis.

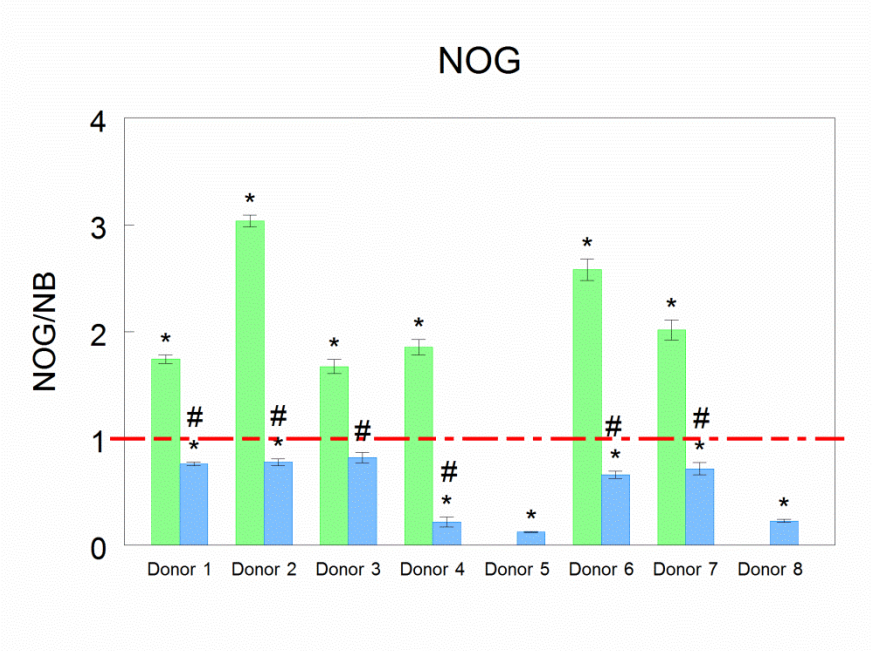


Figure 7. The expression of mRNA levels in patients with coronal craniosynostosis of NOG.

**II.A mRNA Analysis of Patients With Metopic Craniosynostosis**

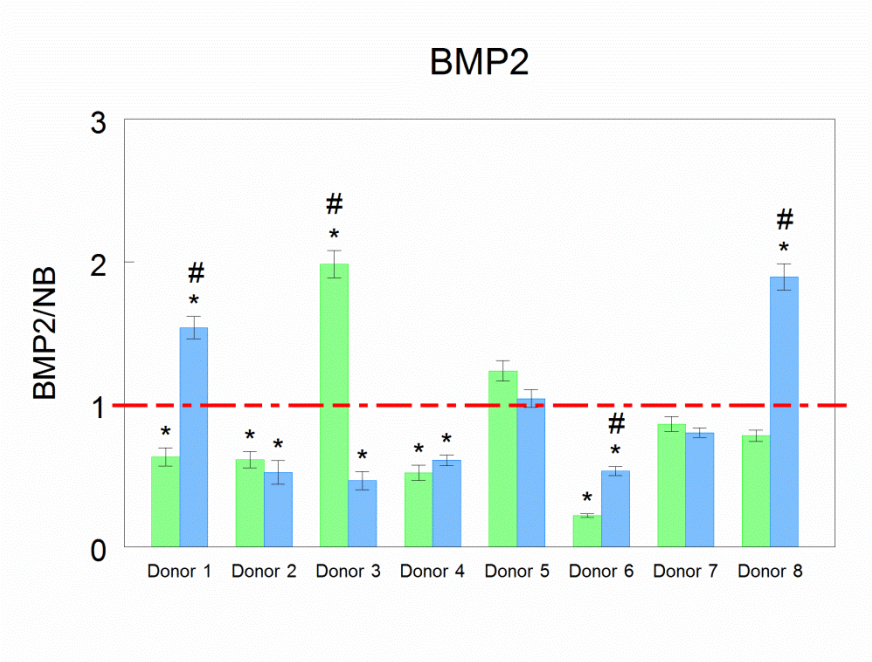


Figure 8. A graph of mRNA levels of BMP2 in patients with metopic synostosis.

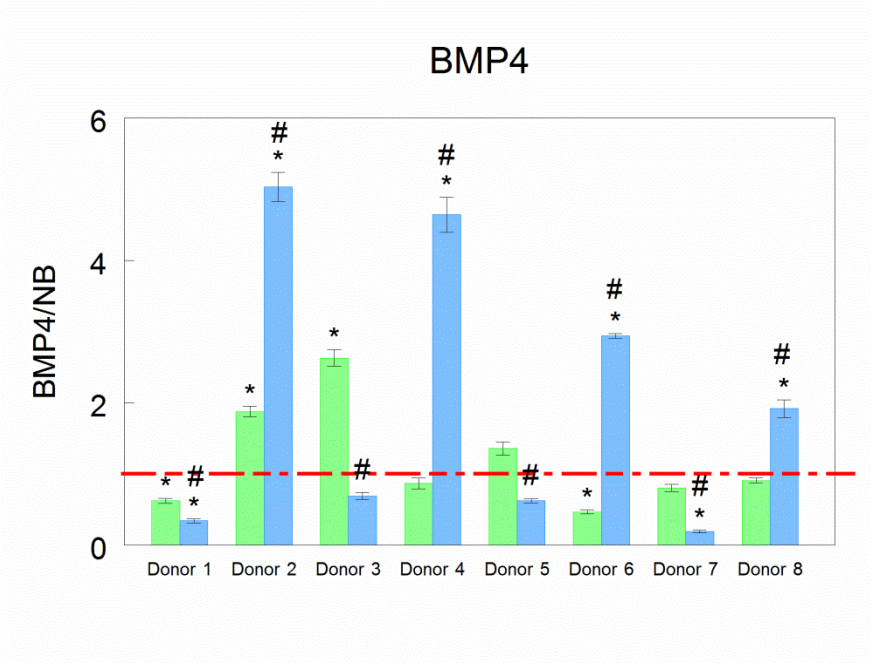


Figure 9. mRNA levels of BMP4 in patients with metopic synostosis.

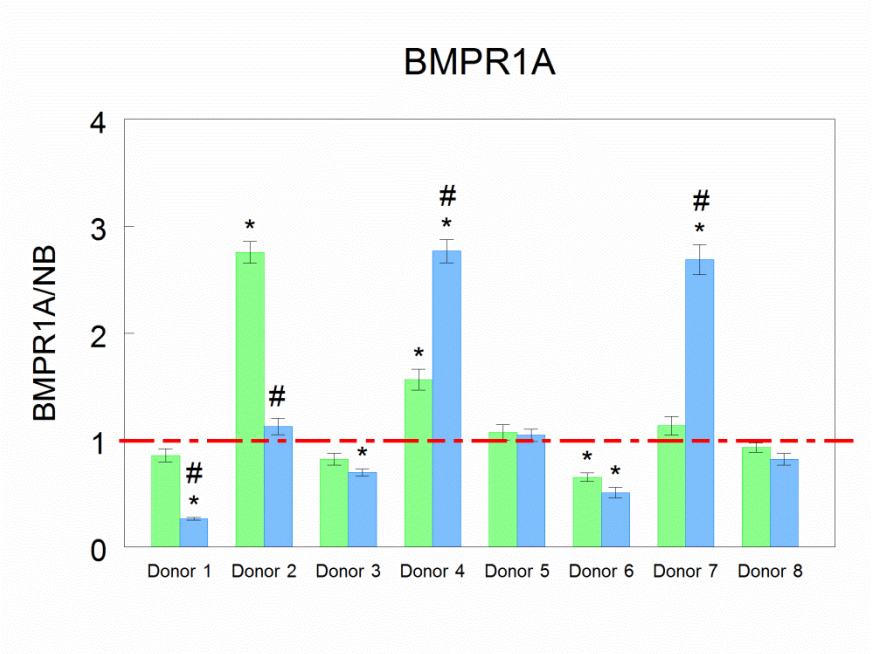


Figure 10. BMPR1A mRNA levels in metopic synostosis.

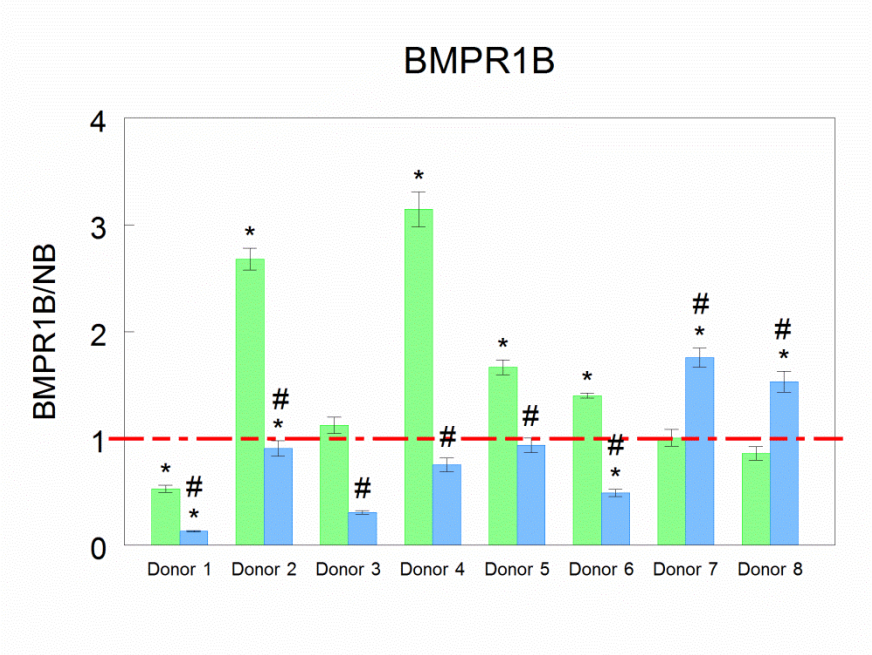


Figure 11. A graph displaying data from mRNA levels of BMPR1B in patients with metopic synostosis.

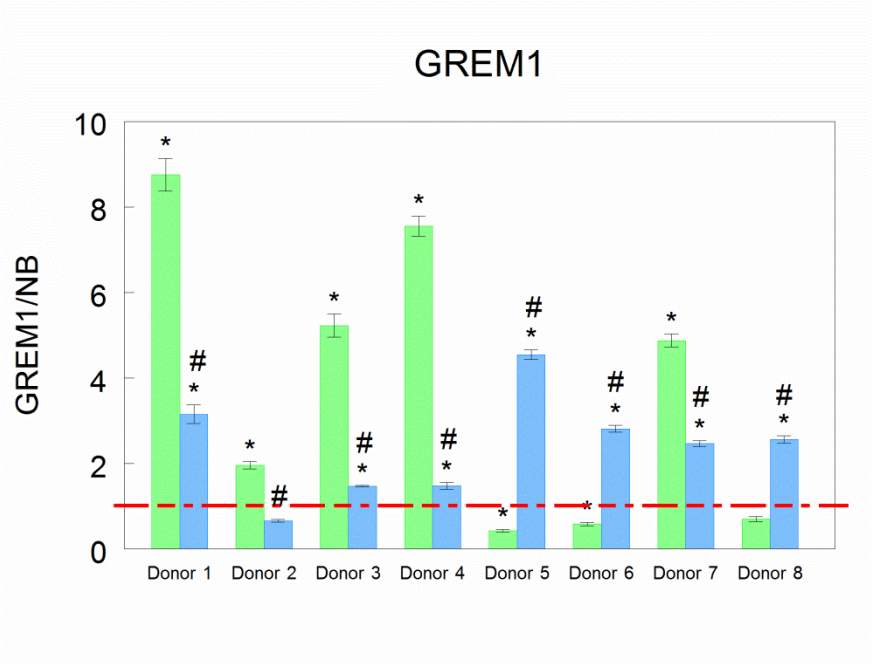


Figure 12. The mRNA levels of GREM1 in metopic synostosis.

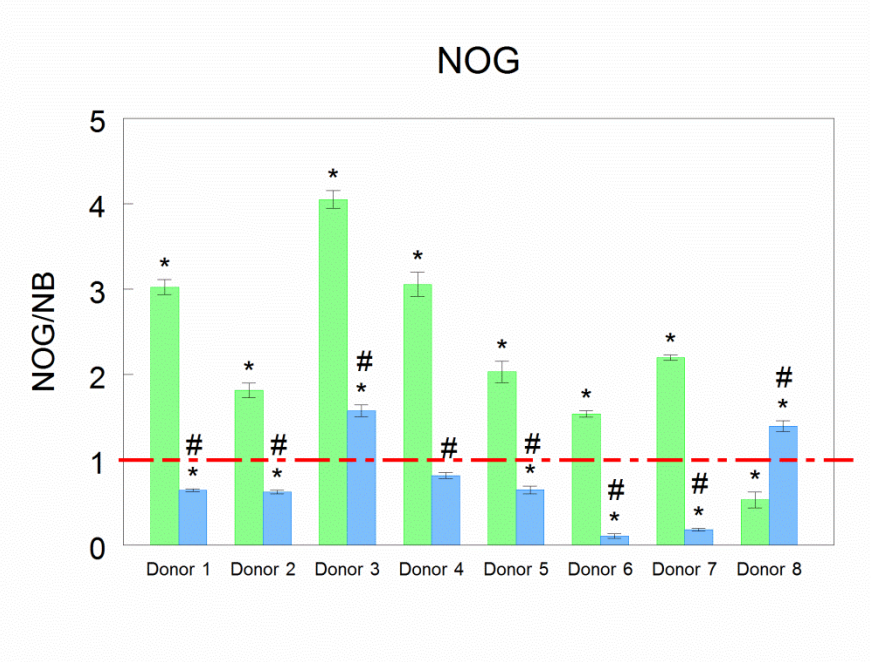


Figure 13. Metopic synostosis analysis of NOG mRNA levels.

**III. mRNA levels of Members of the Bone Morphogenetic Protein Family in Patients with Sagittal Synostosis.**

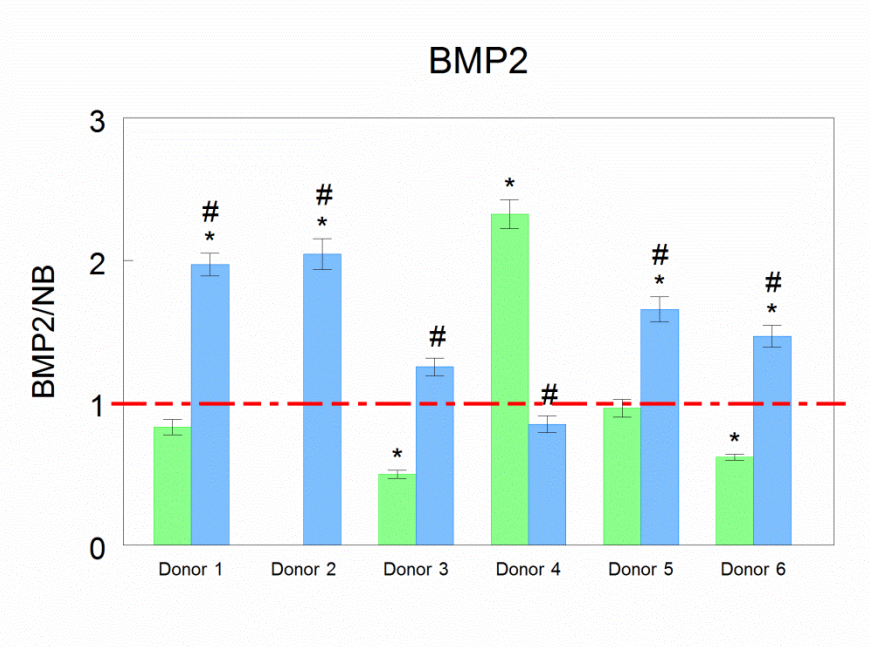


Figure 14. PCR results from an analysis of BMP2 mRNA levels in patients with sagittal synostosis.

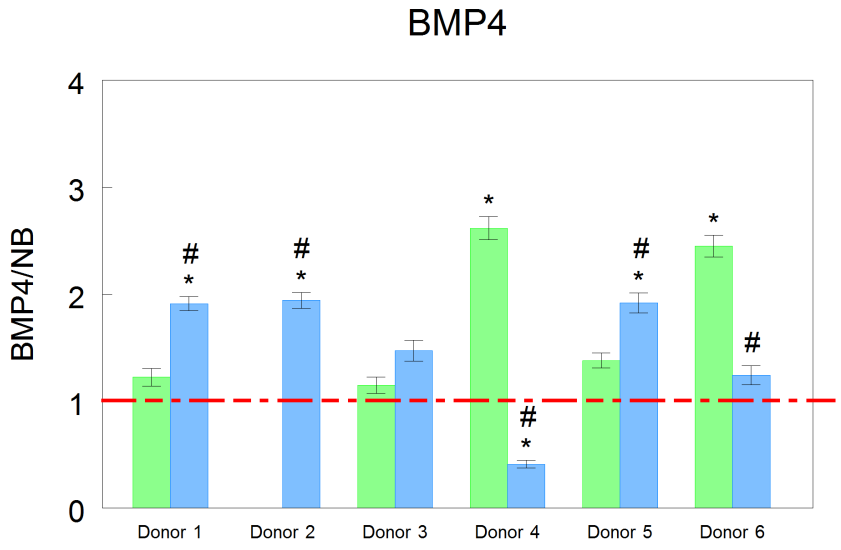


Figure 15. mRNA levels of BMP4 in patients undergoing corrective surgery for sagittal synostosis.

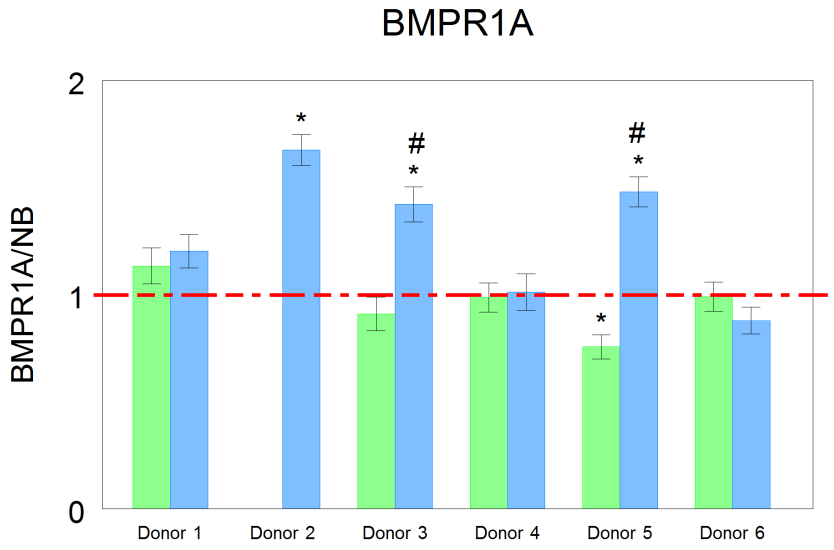


Figure 16. Sagittal Patients' mRNA levels for BMPR1A.

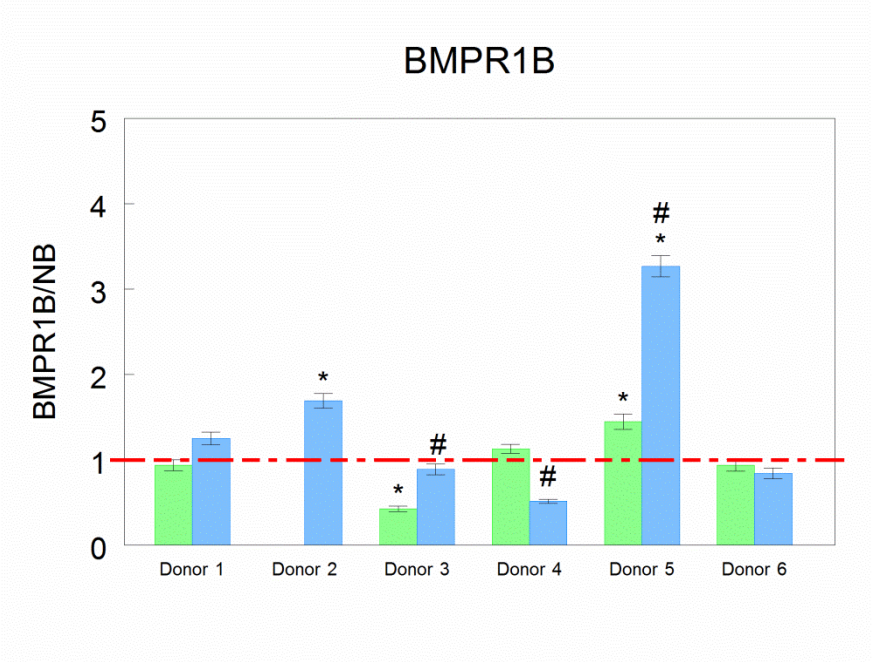


Figure 17. BMPR1B expression in patients with sagittal synostosis.

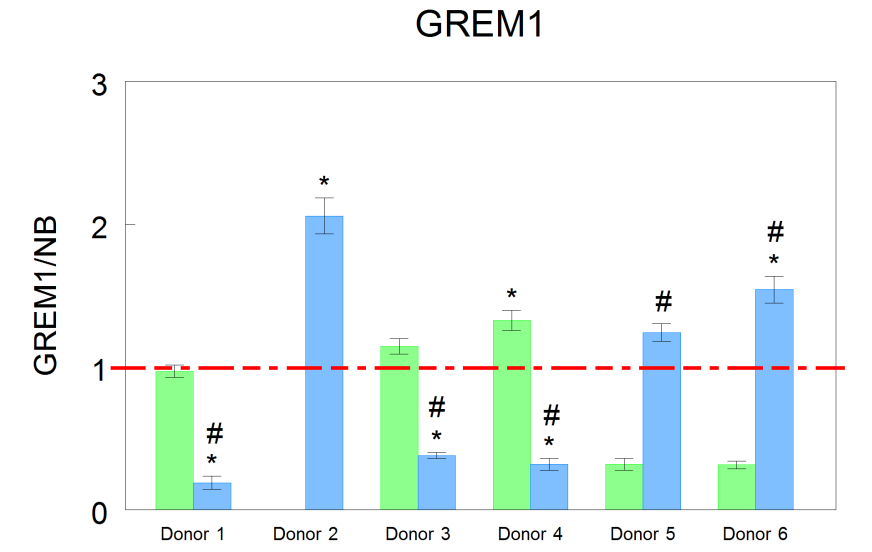


Figure 18. mRNA levels of GREM1 in sagittal synostosis.

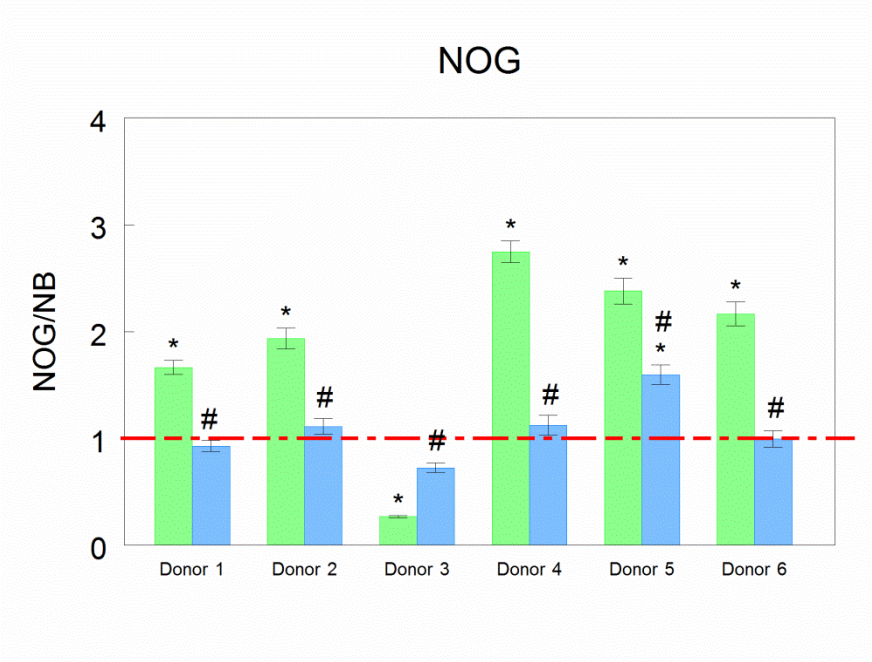


Figure 19. NOG expression in patients with sagittal synostosis.

## IV. Lambdoid Synostosis and mRNA levels for the Bone Morphogenetic Family

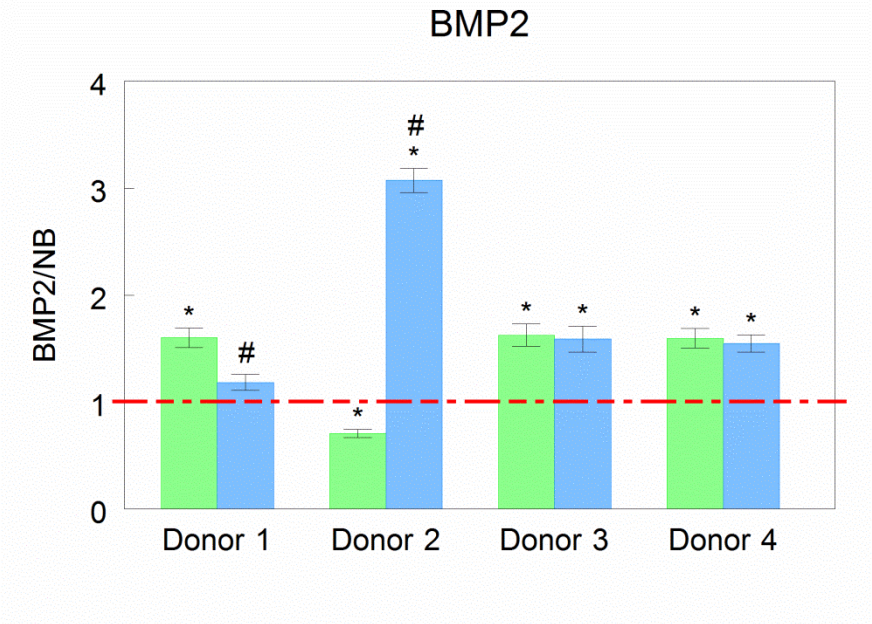


Figure 20. BMP2 mRNA levels in patients with lambdoid synostosis.

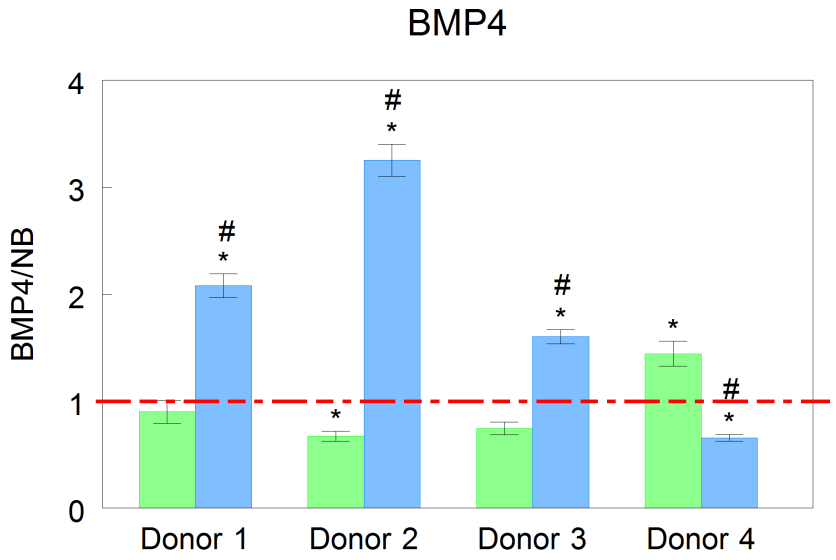


Figure 21. The expression of BMP4 mRNA levels in lambdoid synostosis.

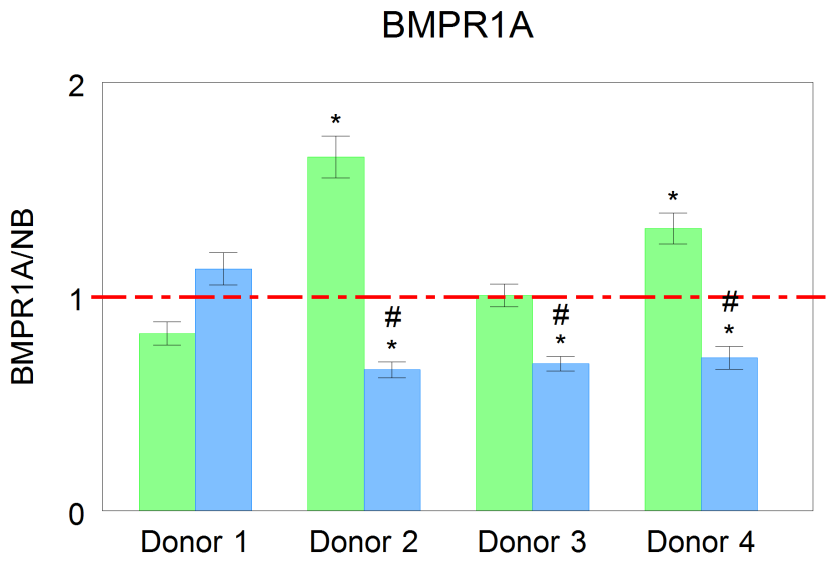


Figure 22. mRNA level expression for BMPR1α in four patients with lambdoid synostosis.

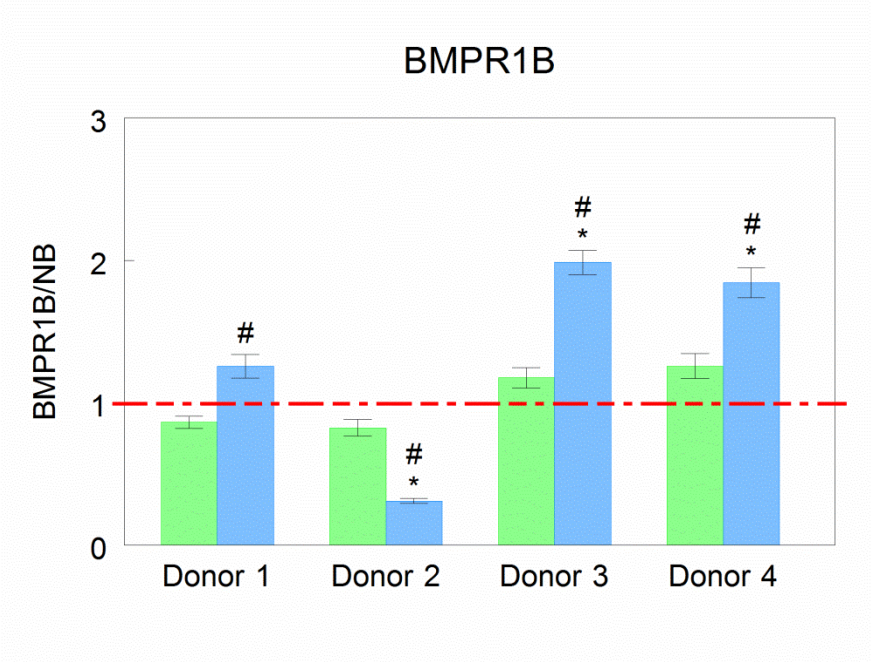


Figure 23. BMPR1β mRNA levels in lambdoid synostosis.

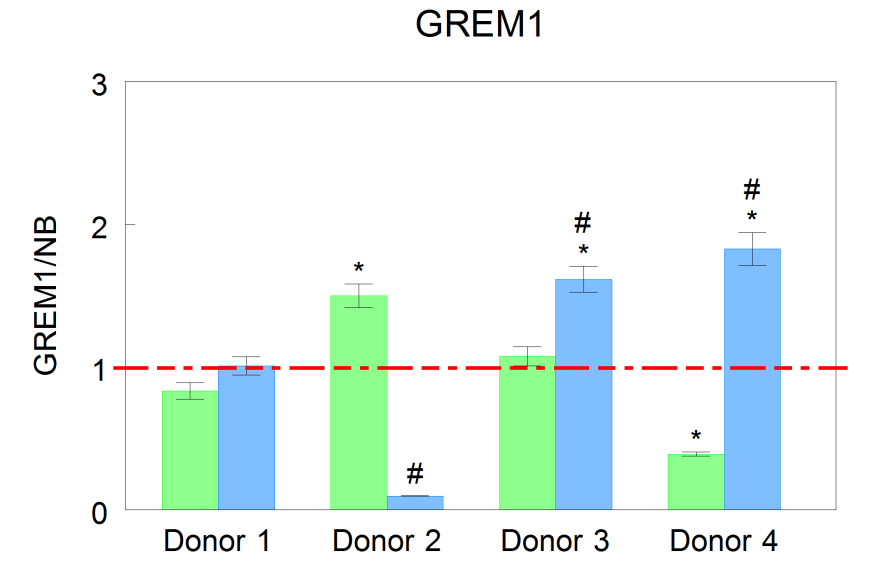


Figure 24. Lambdoid synostosis' mRNA levels in GREM1

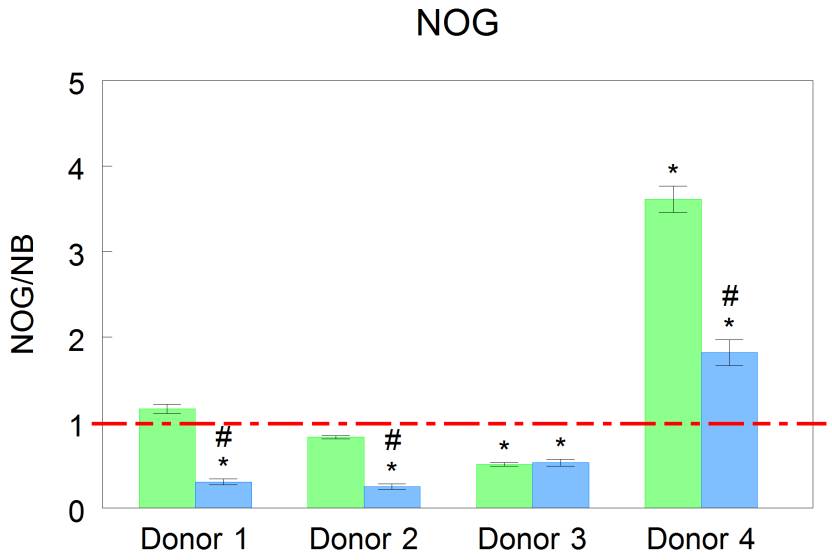


Figure 25. Expression of mRNA of NOG in lambdoid synostosis.

# APPENDIX B: SUPPLEMENTARY TABLES

## I. Primer design

| Gene | Primer sequence |
|---|---|
| BMP2 | F: GCG-TGA-AAA-GAG-AGA-CTG-C<br>R: CCA-TTG-AAA-GAG-CGT-CCA-C |
| BMP4 | F: ACG-GTG-GGA-AAC-TTT-TGA-TGT-G<br>R: CGA-GTC-TGA-TGG-AGG-TGA-GTC |
| BMPR1A | F: CAA-GAG-GCA-TCT-CAA-GCA-GCA-G<br>R:CAG-ACC-CAC-TAC-CAG-ACC-TTT-G |
| BMPR1B | F: AAG-GCT-CAG-ATT-TTC-AGT-GTC-G<br>R: TTC-AAT-GGA-GGC-AGT-GTA-GGG |
| GREM1 | F: GCA-GGG-TGG-GTG-AAC-TTT-ATT-G<br>R: AGG-AGG-CTG-AGA-AGA-TAC-AAG-G |

Supplementary Table 1. A table of primer sequences. The primer for *Noggin* was ordered from a global gene design from Qiagen.

## II. Patient Histories

| **Coronal Synostosis** | |
|---|---|
| Number of male patients. | 1 |
| Number of female patients. | 7 |
| Syndromic cases including familial histories of syndromes. | 5 |
| Average age at operation. | 8.88±2.64 months |

Supplementary Table 2. A description of the patient history for coronal synostosis.

| **Metopic Synostosis** | |
|---|---|
| Number of male patients. | 8 |
| Number of female patients. | 0 |
| Syndromic cases including familial histories of syndromes. | 2 |
| Average age at operation. | 11.43±8.09 months |

Supplementary Table 3. A table with patient history for metopic synostosis.

| Sagittal Synostosis | |
|---|---|
| Number of male patients. | 5 |
| Number of female patients. | 1 |
| Syndromic cases including familial histories of syndromes. | 0 |
| Average age at operation. | 6.17±6.49 months |

Supplementary Table 4. Patient history of surgeries on sagittal synostosis.

| Lambdoid Synostosis | |
|---|---|
| Number of male patients. | 0 |
| Number of female patients. | 4 |
| Syndromic cases including familial histories of syndromes. | 1 |
| Average age at operation. | 12.75±5.62 months |

Supplementary Table 5. A table of patient history for lambdoid synostosis.

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